Inhibition of plasminogen activation by apolipoprotein(a): Role of carboxyl-terminal lysines and identification of inhibitory domains in apolipoprotein(a)

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Running footnote: Apo(a) inhibits plasminogen activation on vascular cells

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Abstract Apolipoprotein(a) [apo(a)]\(^1\), the distinguishing protein component of lipoprotein(a), exhibits sequence similarity to plasminogen and can inhibit binding of plasminogen to cell surfaces. Plasmin generated on the surface of vascular cells plays a role in cell migration and proliferation, two of the fibroproliferative inflammatory events that underlie atherosclerosis. The ability of apo(a) to inhibit pericellular plasminogen activation on vascular cells was therefore evaluated. Two isoforms of apo(a), 12K and 17K, were found to significantly decrease tPA-mediated plasminogen activation on HUVECs and THP-1 monocytes and macrophages. Lp(a) purified from human plasma decreased plasminogen activation on THP-1 monocytes and HUVECs but not on THP-1 macrophages. Removal of kringle V or the strong lysine binding site in kringle IV\(_{10}\) completely abolished the inhibitory effect of apo(a). Treatment with carboxypeptidase B (CpB) to assess the roles of carboxyl-terminal lysines in cellular receptors lead in most cases to decreases in plasminogen activation as well as plasminogen and apo(a) binding; inhibition of plasminogen activation by apo(a) was unaffected, however. Our findings directly demonstrate that apo(a) inhibits pericellular plasminogen activation in all three cell types, although binding of apo(a) to cell-surface receptors containing carboxyl-terminal lysines does not appear to play a major role in the inhibition mechanism.

**Supplementary key words:** lipoprotein(a), atherosclerosis, fibrinolysus, tissue-type plasminogen activator, carboxypeptidase B, thrombin-activatable fibrinolysis inhibitor (TAFI)
Lipoprotein(a) [Lp(a)] was first discovered in 1963 by Kåre Berg (1) and has since been under intense investigation in both basic research and clinical studies (2). Lp(a) has a complex structure consisting of an LDL moiety, which contains apoB-100, and the unique glycoprotein apolipoprotein(a) [apo(a)] (3). The levels of Lp(a) vary over 1000 fold within the human population ranging from less than 1 to greater than 100 mg/dL (4). Lp(a) possesses both proatherogenic and prothrombotic properties due to the LDL-like moiety and apo(a) component of the lipoprotein respectively (5,6). Elevated plasma concentrations of Lp(a) have been identified as an independent risk factor for cardiovascular diseases such as coronary heart disease, ischemic stroke, and peripheral arterial disease (7-11). Lp(a) has also been associated with purely thrombotic events, such as venous thromboembolism (12). The molecular cloning of a cDNA encoding apo(a) revealed that it contains remarkable homology to the fibrinolytic zymogen plasminogen (13).

Apo(a) contains repeated copies of a sequence similar to that of plasminogen kringle IV (KIV) followed by sequences similar to the kringle V (KV) and protease domain of plasminogen (13). The kringle IV-like domain in apo(a) can be further classified into ten subtypes (KIV_1 to KIV_{10}), according to differences in amino acid sequence. The number of KIV_2 repeats determines the isoform size heterogeneity of apo(a) (14,15) with the number of repeats ranging from 3 to >30 (16). The protease domain in apo(a), unlike that of plasminogen, does not exhibit plasmin-like activity and is not proteolytically active (17). Both apo(a) and plasminogen contain lysine binding sites which mediate their respective interactions with various cell surface receptors or the fibrin surface. Apo(a) KIV types 5 to 8 each contain a weak lysine-binding site, with KIV types 7 and 8 forming a noncovalent association with apoB-100 (18-20). This precedes covalent formation of the Lp(a) particle by facilitating disulfide bond formation between a free
cysteine in KIV_9 of apo(a) and the C-terminus of apoB-100 (21,22). Apo(a) KIV_{10} contains a strong lysine-binding site that resembles the lysine-binding site present in plasminogen kringle 4 (23). Apo(a) may therefore potentially interfere with plasminogen binding to or activation on vascular cells.

Plasminogen is the zymogen form of the serine protease plasmin. The active enzyme plays a key role in the processes of fibrinolysis, cell migration and proliferation, angiogenesis, and inflammation (24-29). Plasminogen activation is mediated through proteolytic cleavage of Arg^{561}-Val^{562} of plasminogen by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). This process occurs more readily on the cell surface and following conversion of native Glu-plasminogen to Lys-plasminogen (30-32). Apo(a) has been shown to inhibit plasminogen activation on the fibrin surface through KV and the strong lysine-binding sites in KIV type 10 (33). Moreover, apo(a) directly inhibits Glu- to Lys-plasminogen conversion on the fibrin surface with critical roles identified for kringle IV types 5-9 and KV (34). An apo(a)-mediated decrease in plasmin generation, dependent on apo(a) KIV_9, has also been shown in the context of smooth muscle cells (SMCs) which results in the inhibition of activation of transforming growth factor β (TGF- β) (35); this leads to the stimulation of SMC migration and proliferation (35-37).

The strong lysine-binding site present in plasminogen allows for its binding and subsequent activation to occur on various receptors containing carboxyl-terminal lysines including annexin A2 A100A10 heterotetramer, enolase-1, and Plg-R_{KT} (38). For example, treatment of monocyteid cells with carboxypeptidase B (CpB), which removes carboxyl terminal lysines, leads to a decrease of 64% for plasminogen binding and greater than 95% for plasminogen activation (39). On catecholaminergic cells, binding of plasminogen decreased
following CpB treatment by approximately 70% whereas a decrease of 90% was observed for
plasminogen activation (40). In another study, binding of plasminogen to human umbilical vein
endothelial cells (HUVECs) was also inhibited by ε-aminocaproic acid (ε-ACA), a lysine
analogue, by 70-80% (29).

CpB and active thrombin-activatable fibrinolysis inhibitor (TAFIa) have also been shown
to reduce plasminogen activation by 80% on the annexin A2 S100A10 heterotetramer in vitro
(41). In a rat primary hepatocyte model, TAFI knockdown increased the amount of plasmin
formed on the cells and promoted proliferation, suggesting the ability of TAFIa to decrease
plasminogen binding to hepatocytes (42). Previous studies have suggested that apo(a) may
inhibit pericellular plasminogen activation based on the observation that apo(a) decreases
plasminogen binding to endothelial cells and monocytes (43). Moreover, Lp(a) was reported to
inhibit the generation of plasmin activity on endothelial cell surface and not in the fluid phase
(44). The effect of apo(a) on plasminogen activation on vascular cells has not otherwise been
explored. Most notably, the role of CpB with respect to the effect of apo(a) and plasminogen
binding and activation has not been shown for relevant vascular and blood cells such as
endothelial cells, monocytes and macrophages.
METHODS

Construction, expression, and purification of recombinant apo(a)

The construction and expression in human embryonic kidney (HEK293) cells of the r-apo(a) variants utilized in this study (KIV<sub>5-8</sub>, 12K, 12ΔV, 17K, 17KΔLBS<sub>10</sub>, and 17KΔV) has been previously described (17,33,34,45-48). Variants were purified from conditioned medium, harvested from stably-expressing cell lines, using lysine-Sepharose affinity chromatography as previously described (17,33,34,45-48). Protein concentrations were determined spectrophotometrically (17,33,34,45-48) and assessed for purity by SDS-PAGE under non-reduced and reduced conditions followed by silver staining.

Isolation of Lp(a)

Blood was collected from one healthy volunteer homozygous for a 16K apo(a) isoform. Blood was collected into BD Vacutainer blood collection tubes containing sodium polyanethol sulfonate and acid citrate dextrose. Plasma was obtained through centrifugation of whole blood at 3,000 × g for 15 min at 4°C. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mM and the plasma was rotated at 4°C for 15 min. The plasma was adjusted to a density of 1.063 g/mL with solid sodium bromide and centrifuged at 45,000 × g for 24 hrs at 6°C. The top fraction was removed and the infranatant was adjusted to a density of 1.21 g/ mL with solid sodium bromide. The sample was centrifuged at 45,000 g for 24 hrs at 6°C and the top layer was isolated and extensively dialyzed against 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.01% NaN<sub>3</sub>, and 0.01% EDTA (Buffer A). The sample was then subjected to gel-filtration chromatography over Sepharose CL-4B (Sigma-Aldrich) (2.5 cm × 80 cm column).
using buffer A containing 0.1% (v/v) Tween 20 and 0.1 M proline. Fractions were collected and samples with an absorbance over 0.1 at 280 nm were subjected to western blot analysis using an anti-apo(a) antibody to determine Lp(a) containing fractions. Samples containing Lp(a) were pooled and diluted 3-fold with distilled water and loaded onto a DEAE-Sepharose Fast Flow (Pharmacia) ion exchange column (2.5 × 3 cm column). Lp(a) was eluted with an NaCl concentration gradient (50 to 300 mM NaCl in 20 mM Tris-HCl, pH 7.4). Lp(a) containing fractions, as determined by western blot analysis, were pooled and dialyzed against HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4, 150 mM NaCl). Concentration was determined by bicinchoninic acid assay (Pierce) using BSA as a standard. The integrity of the purified Lp(a) was assessed by SDS-PAGE under non-reducing and reducing conditions followed by silver staining.

**Cell culture**

HUVECs were obtained from Clonetics and grown in EGM Complete Medium (Clonetics). All HUVEC experiments were conducted using cells at passage five. Human acute monocytic leukemia (THP-1) cells were grown in RPMI complete growth medium 1640 (GIBCO) adjusted to contain 4.5 g/L glucose (Sigma-Aldrich), 10 mM HEPES (Fisher Scientific), 1.0 mM sodium pyruvate (GIBCO), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) and supplemented with 10% FBS (GIBCO) and 1% antibiotic-antimycotic (GIBCO). Cells were maintained at a density of 0.3-1 × 10^6 cells/mL and subculturing was performed by centrifuging cells at 100 × g for 5 min and resuspending in the appropriate volume of fresh medium. PMA (Sigma-Aldrich) was added to THP-1 cells at a final concentration of 0.1 μM for 72 hours to differentiate them into macrophage-like cells.
**Plasminogen activation assay**

HUVECs were grown to confluency in a 96-well microtiter plate (Corning). THP-1 cells were seeded at a density of 150 000 – 200 000 cells per well and differentiated for 72 hours with 0.1 µM PMA. THP-1 monocytes were seeded at a density of 200 000 cells per well. Cells were washed three times with HBS containing 0.4% (w/v) BSA (HBS-BSA) prior to adding reaction mixture. Reaction mixtures contained: 60 nM plasma derived plasminogen (purified as per (49)), 15 nM tPA (Alteplase; Kingston General Hospital Pharmacy), H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (Bachem), and various concentrations of apo(a) (0, 100, 400, or 800 nM) in HBS-BSA. RPMI complete growth medium minus FBS and antibiotic and supplemented with 0.4% (w/v) BSA (RPMI-0.4%BSA) was used instead of HBS-BSA for THP-1 macrophages. Control experiments were conducted in the absence of t-PA. Plasmin formation was monitored over 2 hours at 37°C at an excitation wavelength of 370 nm and an emission wavelength of 470 nm and emission cutoff filter of 455 nm using a plate reading fluorescence spectrometer (SpectraMax M5®, Molecular Devices). The rate of plasminogen activation was taken as the initial slope of the plot of RFU against min² from 10 to 40 mins. Rates obtained for each individual experiment performed in triplicate were normalized to the rate of plasminogen activation in the absence of apo(a). Plasminogen activation in the absence of cells was performed with the same reaction mixture described above. Plasminogen activation with Lp(a) was conducted as above with minor changes; 25 nM of plasma derived plasminogen, instead of 60 nM, was utilized with 75 nM of Lp(a) or apo(a).

**Cell binding assay**
Assays utilized purified r-apo(a) fluorescently labeled using Alexa Fluor 488 protein labeling kit (Invitrogen) or fluorescein isothiocyanate-labeled human plasminogen (Molecular Innovations). Briefly, cells were washed three times with HBS-BSA and fluorescently labeled plasminogen or r-apo(a), at a concentration of 400 nM, was incubated with cells for one hour at 37°C. Following incubation, cells were washed twice with HBS-BSA and the fluorescence was measured at an excitation wavelength of 494 nm and emission wavelength of 519 nm and cutoff of 515 nm using a plate reading fluorescence spectrometer. For THP-1 macrophages, the buffer used was RPMI-BSA instead of HBS-BSA. Experiments in which two to three acid wash steps (0.2 M acetic acid pH 2.5, 0.5 M NaCl) were included prior to measuring the fluorescence showed that at least 80% of the labeled proteins were surface-bound (as opposed to internalized; Suppl. Fig. 1). Binding of apo(a) and plasminogen to the cells appeared to be specific and reversible (Suppl. Fig. 2).

Carboxypeptidase treatment

CpB (Calbiochem) treatment of HUVECs and THP-1 monocytes was performed in HBS-BSA and for THP-1 macrophages in RPMI-BSA. Cells were washed three times and incubated with CpB for one hour at 37°C. Cells were then washed three times followed by assay of plasminogen activation or apo(a)/plasminogen binding as described above. The activity of CpB was measured using anisylazoformylarginine (Calbiochem).

Statistical Methods

Comparisons between data sets were performed using one-way ANOVA with SPSS version 22 software. Statistical significance was presumed at p<0.05.
RESULTS

Apo(a) directly inhibits plasminogen activation on THP-1 monocytes, THP-1 macrophages, and HUVECs

We examined two different isoforms of apo(a) (12K and 17K) and both were found to inhibit pericellular plasminogen activation in THP-1 monocytes (Fig. 1A,B). Both isoforms exhibit a similar concentration-dependent effect on inhibition. At 800 nM, 12K and 17K inhibited plasminogen activation by 57% and 46% respectively. At 800 nM, 12K was not significantly more effective at inhibiting activation compared to 17K. Removal of kringle V in both 12K and 17K not only abolished the inhibitory effect of apo(a) but, surprisingly, caused an increase in plasminogen activation (up to 3.6- and 2.4-fold, respectively; Fig. 1A,B). Similarly, inactivation of the strong lysine-binding site in apo(a) (17KΔLBS10 variant) led to a maximal 2.1 fold increase in plasminogen activation (Fig. 1A). The weak lysine-binding sites of apo(a), present in the KIV5-8 variant, do not exhibit a significant inhibitory effect at any of the tested concentrations (Fig. 1B). These results indicate that apo(a) can directly inhibit pericellular plasminogen activation in THP-1 monocytes and imply roles for kringle V and the strong lysine-binding site in KIV type 10 in mediating this effect.

Comparing plasminogen activation in the presence or absence of THP-1 monocytes revealed a 74% decrease in activation in the absence of cells, and this rate was not affected by 17K, 12K or KIV5-8 (Fig. 1A,B). However, 17KΔLBS10, 17KΔV, and 12KΔV shows a significant increase in plasminogen activation with increasing concentration similar to that in the presence of cells, suggesting that the cell-independent stimulation of plasminogen activation by
these variants largely accounts for the increase in plasminogen activation occurring in the presence of cells.

Treatment of THP-1 cells for 72 hours with a phorbol ester results in the differentiation of these cells into macrophage-like cells (50). Apo(a) inhibits plasminogen activation on THP-1 macrophages in a dose-dependent manner, with a maximal inhibition of 57% and 65% for 12K and 17K, respectively, observed at 800 nM (Fig. 1C,D). At 800 nM, 12K and 17K were not significantly different in its ability to inhibit activation. Both the strong lysine-binding site in KIV type 10 of 17K (17KΔLBS10) (Fig. 1C) and the kringle V domain (12KΔV) (Fig. 1D) are required for the inhibitory effect of apo(a); as with the THP-1 monocytes, both of these variants resulted in increased, rather than decreased, plasminogen activation. For the 17KΔLBS10 and 12KΔV variant, this increase again can be ascribed to stimulation of cell-independent plasminogen activation (Fig. 1C,D). In the absence of THP-1 macrophages, the rate of plasminogen activation was significantly lower (71%) and was unaffected by the presence of 12K and 17K, as was the case for THP-1 monocytes.

Similar experiments were also performed with HUVECs, and with similar results. Apo(a) directly inhibits pericellular plasminogen activation on the surface of HUVECs. A maximal inhibitor effect of 48%, for 12K, and 58%, for 17K was observed at a concentration of 800 nM (Fig. 1E,F). At 800 nM, 12K was not significantly more effective at inhibiting activation compared to 17K. As with THP-1 monocytes and macrophages, loss of the strong lysine-binding site in KIV10 or the loss of KV resulted in a cell-independent increase in plasminogen activation (Fig. 1E,F).

Taken together, the effect of apo(a) on cell-dependent plasminogen activation is remarkably similar for all three cell types tested; moreover, the effect of loss or mutation of
specific domains of apo(a) is consistent in all three cell types. These results indicate a potential common mechanism through which apo(a) inhibits pericellular plasminogen activation in each cell type.

To demonstrate that activation of plasminogen observed was dependent on the added tPA and plasminogen and not from the cells themselves or contamination of the r-apo(a), experiments were conducted in the absence of tPA or plasminogen. Omission of either of these components virtually eliminated detectable plasminogen activation in the presence of all three cell types, either in the presence or absence of various r-apo(a) variants (Fig. 2).

Lp(a) inhibits plasminogen activation on THP-1 monocytes and HUVECs but not on THP-1 macrophages

We examined the ability of Lp(a), purified from human plasma, to inhibit pericellular plasminogen activation. Experiments were conducted using 25 nM plasminogen and 75 nM of Lp(a) or apo(a). Lp(a) and 17K significantly inhibited plasminogen activation by 15% and 35% on THP-1 monocytes respectively (Fig 3A). However, Lp(a) did not inhibit plasminogen activation on THP-1 macrophages whereas a decrease of 24% was observed with 17K (Fig 3B). Conversely, both Lp(a) and 17K could inhibit plasminogen activation to the same extent, 30% and 28% respectively, on HUVECs (Fig 3C). As observed above, plasminogen activation was largely abolished in the absence of tPA or cells, and was not altered with the addition of Lp(a) or 17K (Fig. 3)

Role of carboxyl terminal lysines in cell-dependent plasminogen activation and its inhibition by apo(a)
It has been previously reported that carboxypeptidase B (CpB) treatment of U937 monocytoid cells reduced plasminogen binding by 64% and cell-enhanced plasminogen activation by 95% (39). These results have been interpreted to mean that plasminogen binding to these residues on cell-surface receptors is required for the acceleration of plasminogen activation by cells. Treatment of THP-1 monocytes with various concentrations of CpB ranging from 0.1 to 100 U/mL results in a significant decrease in plasminogen activation (by a modest 16%) at 100 U/mL (Fig 4A,B). Addition of 17K, at 400 nM, at any concentration of CpB results in a decrease in plasminogen activation at all concentrations of CpB; CpB no longer has any significant effect at any concentration in the presence of 17K (Fig. 4A). Similar results were observed for THP-1 macrophages (Fig. 4C), except that CpB treatment had a greater effect of plasminogen activation, with significant decreases occurring at 50 and 100 U/mL both in the presence and absence of 17K (Fig. 4C). Receptors with carboxyl terminal lysines also influence plasminogen activation on HUVECs as a decrease of 24% is observed following treatment with 100 U/mL CpB; a decrease in plasminogen activation owing to CpB was also observed in the presence of 17K although statistical significance was not reached (Fig. 4E).

**Role of carboxyl terminal lysines in plasminogen and apo(a) binding by cells**

To attempt to rationalize the effect of CpB on the plasminogen activation of the surface of cells, and its inhibition by apo(a), we examined the ability of plasminogen and apo(a) to bind to THP-1 monocytes, macrophages, and HUVECs, after CpB treatment or in the presence of the lysine analogue ε-ACA. Plasminogen and apo(a) binding (using 400 nM) decreased by 18% and 51% respectively following CpB treatment (100 U/mL) on THP-1 monocytes (Fig. 4B). Binding of plasminogen and apo(a) decreased by 84% and 79% respectively with the addition of 200 mM
 ε-ACA (Fig. 4B). Similarly, in THP-1 macrophages, the addition of ε-ACA reduced plasminogen and apo(a) binding by 91% and 73% respectively (Fig. 4D). However, a more modest decrease in plasminogen and apo(a) is observed following CpB treatment at 100 U/mL (9% and 13% respectively) (Fig. 4D). Plasminogen binding to HUVECs also decreases following CpB (100 U/mL) treatment and the addition of ε-ACA by 29% and 75% respectively (Fig. 4F). Surprisingly, however, apo(a) binding actually increases by 25% and 3% following CpB (100 U/mL) treatment and the addition of ε-ACA respectively (Fig. 4F). These results indicate that apo(a) binding is partially mediated through receptors with carboxyl terminal lysines on THP-1 macrophages and monocytes but not on HUVECs. However, since CpB treatment had little or no effect on the ability of apo(a) to inhibit plasminogen activation, these lysine-dependent binding events play a minor role, if any, in inhibition of plasminogen activation by apo(a).
DISCUSSION

It is generally accepted that apo(a) can inhibit pericellular plasminogen activation on vascular and blood cells, although the mechanism is not understood. As well, the domains in apo(a) which may potentially mediate this inhibitory effect and the relative role of apo(a) in various cell types is unknown. The present study contributes to the understanding of the role of apo(a) in inhibiting pericellular plasminogen activation on vascular and blood cells, specific domains in apo(a) which mediate this inhibitory effect, as well as the role of carboxyl terminal lysines in plasminogen activation and plasminogen and apo(a) binding.

In this study, we have provided direct evidence that (i) apo(a) inhibits pericellular plasminogen activation on HUVECs, THP-1 monocytes, and THP-1 macrophages; (ii) the kringle V domain and strong lysine-binding site in kringle IV type 10 play a crucial role in inhibiting activation whereas kringle 5-8 does not; (iii) Lp(a), at ~7 mg/ dL or 75 nM, can inhibit plasminogen activation on THP-1 monocytes and HUVECs but not on THP-1 macrophages (iv) plasminogen binding to the cells and cell-dependent plasminogen activation are dependent to a partial extent on receptors containing carboxyl-terminal lysines on all cell types; (v) apo(a) binding to THP-1 monocytes and macrophages is partially dependent of these receptors, but apo(a) binding to HUVECs is independent of these receptors and of lysine binding in general; and (vi) the binding by apo(a) to carboxyl-terminal lysine-containing sites does not appear to account for its ability to inhibit plasminogen activation.

A previous study has shown that Lp(a)/ apo(a) can compete with plasminogen for binding on the endothelial cell surface and hence inhibit plasminogen activation (44). Binding of plasminogen to the cell surface initiates pericellular plasminogen activation through interaction
with its respective plasminogen activators: tPA and uPA. Both tPA and uPA have been shown to be expressed in a variety of cell types, including endothelial cells for tPA (51) and lung, kidney, and several tumour cell lines for uPA (52,53). It has been shown that tPA can directly bind to various cell types other than endothelial cells, such as platelets, monocytes, and monocytoid cells (30,39,54,55). Lp(a) was reported to inhibit plasminogen activation on the surface of resting platelets by inhibiting plasminogen and tPA binding (56). Therefore, the effects of apo(a) and Lp(a) on tPA-mediated pericellular plasminogen activation on vascular cells were evaluated.

Plasminogen receptors are a heterogeneous and ubiquitously present group of cell surface proteins (38). Plasminogen receptors have been shown to contain carboxyl-terminal lysine residues that bind plasminogen (38). Removal of these carboxyl-terminal lysines destroys the ability of this subset of receptors to bind to plasminogen and support its activation (38). However, not all plasminogen receptors contain a carboxyl-terminal lysine, with examples of this type being actin, amphoterin, αVβ3, αMβ2, and αIIbβ3 (57). Binding of plasminogen to these receptors presumably converts plasminogen from its native closed conformation into a more open and highly activatable form (38). The initial plasmin generated on the cell surface begins to catalyze the positive feedback mechanism of converting Glu- to Lys-plasminogen, the latter of which is itself more readily activatable (32). Furthermore, the plasmin generated on the surface of the cells is protected from its natural inhibitor α2-antiplasmin (31). In the context of fibrin, carboxyl terminal lysines have been shown to play a crucial role in regulating plasminogen activation (25,58). Hence, the role of carboxyl-terminal lysines on the cell surface was also of interest in this study.

Our studies, for the first time, have directly demonstrated that apo(a) is capable of inhibiting pericellular plasminogen activation. We showed this to be the case with two different
recombinant apo(a) species, 12K and 17K, representing physiologically-relevant apo(a) isoforms. Interestingly, the 12K and 17K were similar in their ability to inhibit pericellular plasminogen activation. This suggests that the number of KIV type 2 repeats do not dictate the level of inhibition which is in agreement with the findings of Hancock and co-workers in the context of fibrin (33). On the other hand, studies using a wider array of apo(a) sizes in the context of an intact Lp(a) particle have reported an inverse relationship between isoform size and the ability of Lp(a) to inhibit fibrinolysis and interfere with plasminogen binding to fibrin (59,60), while larger Lp(a) isoforms were found to bind with less affinity to THP-1 monocytes (61). Therefore, additional studies are required to assess the possibility that inhibition of pericellular plasminogen activation by apo(a)/Lp(a) is also isoform-size dependent.

The concentration of circulating plasminogen in plasma is 2.2 μM (62) whereas the concentration of Lp(a) varies from 1 to greater than 100 mg/dL, which is approximately equal to 0.01 to 1 μM (4). The molar concentration of plasminogen circulating in the blood is generally greater than that of apo(a); however apo(a) has been shown to accumulate within atherosclerotic lesions and carotid plaques, likely due to its ability to interact with a wide variety of extracellular matrix elements (63-65). Importantly, we showed that the ability of apo(a) and Lp(a) to inhibit pericellular plasminogen activation was strictly dependent on the presence of cells (Figs. 1, 3). This likely reflects the ability of apo(a) to influence binding of plasminogen and/or tPA to the cells, although in our own binding studies we were not able to conclusively determine the extent to which apo(a) and plasminogen compete for binding to the cells (Suppl. Fig. 3).

Previous studies have identified specific domains of apo(a) that mediate its inhibitory effect on plasminogen activation as well as Glu-plasminogen to Lys-plasminogen conversion on the fibrin surface (33,34). Ablation of the strong lysine binding site in kringle IV type 10 was
shown to substantially reduce the inhibitory effect of apo(a) whereas removal of kringle V abolished the effect in plasminogen activation on fibrin (33). Critical roles for kringle IV types 5-9 and kringle V were identified in inhibiting Glu- to Lys-plasminogen conversion on fibrin (34). Interestingly, apo(a) from baboons and rhesus monkeys do not contain kringle V and are not prone to atherosclerosis (66, 67). In the case of baboons, the absence of KV renders the Lp(a) particle incapable of binding to lysine despite the presence of an intact strong LBS in KIV$_{10}$ (66). On the other hand, in our previous studies of SMCs, we found that KIV$_9$, but not the strong LBS in KIV$_{10}$, mediated a decrease in TGF-β activation in a mechanism that was dependent on the presence of plasminogen (and thus, presumably, an effect on plasminogen activation) (35). Therefore, there may be cell-type differences in the mechanisms through which apo(a)/Lp(a) interferes with plasminogen activation. This may in turn reflect different cohorts of plasminogen – or apo(a) – receptors on difference cell types.

We show here that the strong lysine binding site in kringle IV type 10 as well as kringle V are essential for the inhibitory effects of apo(a) on pericellular plasminogen activation on THP-1 monocytes, THP-1 macrophages, and HUVECs. In fact, removal of theses domains had the unexpected effect of enhancing plasminogen activation on the vascular cells tested. We suspect that these effects are related to the ability of apo(a) to bind to plasminogen in solution (46). Indeed, from experiments conducted in the presence and absence of cells, it appears this enhancing effect of 17KΔLBS$_{10}$, 17KΔV and 12KΔV is unaffected by the presence or absence of cells. However, 12K and 17K r-apo(a) do not enhance plasminogen activation in the absence of cells. Accordingly, we must hypothesize that 17KΔLBS$_{10}$ and 12KΔV bind plasminogen in such a way that promotes its solution-phase activation, while intact 12K or 17K do not.
The ability of Lp(a) to inhibiting plasminogen activation was also evaluated (Fig. 3). Lp(a) was found to inhibit plasminogen activation, to the same extent as 17K, on HUVECs and to a lesser extent on THP-1 monocytes. Plasminogen activation was not inhibited with the low concentration of Lp(a) utilized, ~7 mg/dL, on THP-1 macrophages. We were restricted to a relatively low concentration of Lp(a) by the concentration of the preparation. The concentration utilized is less than the cardiovascular risk factor of Lp(a) levels >50 mg/dL and could account for the lack of effect on THP-1 macrophages, possibly due to more rapid uptake of Lp(a) by these cells for example by the apo(a)/Lp(a) receptor identified by Keesler and coworkers (48).

The importance of carboxyl terminal lysine receptors was determined in vascular and blood cells. Overall, CpB treatment had modest but significant decreasing effects on plasminogen activation and on plasminogen and apo(a) binding (Fig. 4), with CpB treatment never decreasing binding or activation to below 50% of control. The exception to this was in HUVECs, where apo(a) bound in a manner completely independent of carboxyl-terminal lysines (Fig. 4F). Addition of the lysine analogue ε-ACA had a more profound effect on plasminogen or apo(a) binding, which may suggest that some binding sites may have represented internal, as compared to carboxyl-terminal, lysine residues. Because of its profound effects on plasminogen conformation, we were unable to assess the effects of ε-ACA on plasminogen activation. Nonetheless, the available data suggest that the inhibition of plasminogen activation by apo(a) is not dependent on carboxyl-terminal lysines, since CpB treatment of the cells had only a small effect, if any, on the extent of the inhibition. If carboxyl-lysines played a role (such as mediating the binding of apo(a), plasminogen, or tPA required for the inhibition) inhibition by apo(a) would have been eliminated, when in fact it was only minimally impacted (Fig. 4A,C,E). Indeed, in HUVECs, where apo(a) is able to inhibit plasminogen activation over 50%, apo(a) binding is
completely independent of carboxyl-terminal lysines (Fig. 4). It is reasonable to hypothesize, then, that if lysines do play a role, they would be internal lysines in cell-surface receptors.

Apo(a) has been shown in this current study to play a crucial role in regulating plasminogen activation on vascular cells attributable to kringle V and the strong lysine-binding site in kringle IV type 10. What remains to be determined are the identities of the receptors that presumably mediate these effects. Moreover, it must be evaluated if the inhibition of plasminogen activation by apo(a) results from competition between these proteins for binding sites on cells and/or is a consequence of the ability of apo(a) to inhibit plasminogen activation via interference with the positive feedback mechanism of Glu- to Lys-plasminogen conversion.
FOOTNOTES

The abbreviations used are: apo(a): apolipoprotein(a); Lp(a): lipoprotein(a); CpB: carboxypeptidase B; KIV: kringle IV; KV: kringle V; tPA: tissue-type plasminogen activator; uPA: urokinase-type plasminogen activator; TGF-β: transforming growth factor β; HUVECs: human umbilical vein endothelial cells; ε-ACA: ε-aminocaproic acid; TAFI: thrombin- activatable fibrinolysis inhibitor; TAFIa: activated TAFI; HBS: HEPES-buffered saline.
REFERENCES


FIGURE LEGENDS

**Fig. 1.** Effect of apo(a) on pericellular plasminogen activation. THP-1 monocytes (A,B), THP-1 macrophages (C,D), or HUVECs (E,F) were incubated with various concentrations of r-apo(a) (from 100 nM to 800 nM) as well as plasma-derived plasminogen (60 nM), tPA (15nM), and H-D-Val-Leu-Lys-AMC; control reactions lacked apo(a) or cells. Plasmin generation was monitored over 2 hours at 37°C and the slope of RFU from 10-40 min vs min² was determined. The data represent the means ± standard deviations from 3 - 16 independent experiments performed in triplicate for cells and 3 - 6 independent experiments for no cells. Asterisks: p<0.05 versus absence of apo(a) on cells; daggers: p<0.05 versus absence of cells.

**Fig. 2.** Plasminogen activation in the absence of tPA on THP-1 monocytes, THP-1 macrophages, and HUVECs. Plasminogen activation experiments were conducted as described in the legend to Figure 1, but in the absence of plasminogen and tPA. A: Effect of plasminogen activation in the absence of plasminogen and tPA for plasminogen alone, 12K, 12KΔV, 17K, 17KΔLBS₁₀, 17KΔV, and KIV5-8 at 400 nM each on THP-1 monocytes. B: Effect of plasminogen activation in the absence of tPA for plasminogen alone, 17K, 17KΔLBS₁₀, and 17KΔV at 400 nM each on THP-1 macrophages and HUVECs. Data represent the means ± standard deviations from 3-7 independent experiments performed in triplicate. In all cases, the relative plasminogen activation was significantly different (p<0.05) from control (in the presence of plasminogen and tPA combined).
Fig. 3. Effect of Lp(a) on plasminogen activation on THP-1 monocytes, THP-1 macrophages, and HUVECs. Plasminogen activation experiments were performed as described in the legend to Figure 1 with slight modifications: 25 nM of plasminogen and 75 nM of Lp(a)/apo(a) was utilized. A: Effect of Lp(a) on plasminogen activation in the presence or absence of THP-1 monocytes and tPA. B: Effect of Lp(a) on plasminogen activation in the presence or absence of THP-1 macrophages and tPA. C: Effect of Lp(a) on plasminogen activation in the presence or absence of HUVECs and tPA. The data represent the means ± standard deviations from 3 - 6 independent experiments performed in duplicate. Asterisks: p<0.05 versus plasminogen alone in the presence of cells and tPA; daggers: p<0.05 versus Lp(a) in the presence of cells and tPA.

Fig. 4. Effect of removal of carboxyl terminal lysines using CpB on plasminogen activation and plasminogen and apo(a) binding. A,C,E: Effect of CpB treatment in plasminogen activation on THP-1 monocytes (A), THP-1 macrophages (C), or HUVECs (E) in the presence or absence of r-apo(a). Cells were washed three times followed by incubation with CpB, at various concentrations, for one hour at 37°C. Cells were then washed three times following which plasminogen activation was measured as described in the legend to Figure 1. Asterisks: p<0.05 versus absence of CpB for plasminogen activation in absence of apo(a); daggers: p<0.05 versus absence of CpB for plasminogen activation in presence of apo(a). B,D,F: Effect on plasminogen and apo(a) binding following CpB treatment of THP-1 monocytes (B), THP-1 macrophages (D), or HUVECs (F). Cells treated with CpB were washed three times and incubated with fluorescently labeled plasminogen or 17K at 400 nM, in the presence or absence of 200 mM ε-ACA for one hour at 37°C. Cells were washed two times and the amount of bound fluorescent proteins was measured using a plate-reading fluorimeter. The data represent the means ±
standard deviations from 4 - 11 independent experiments performed in triplicate for plasminogen activation and 3-7 independent experiments for binding experiments. Asterisks: p<0.05 versus absence of CpB (or ε-ACA) for plasminogen; daggers: p<0.05 versus absence of CpB (or ε-ACA) for apo(a).
Figure 1
Figure 2
Figure 3
Figure 4